Protocol for founder screening and genotyping by fluorescent PCR for ZFN/TALEN/CRISPR-mediated mutagenesis in zebrafish

(Raman Sood, Blake Carrington, MaryPat Jones, 6/24/15)

1. Primer Design:

Since zebrafish are highly heterogeneous, it is best to test the target region for SNPs and in/dels by sequencing a few wildtype fish of the strain you plan to use for mutagenesis. Design primers to amplify 125 to 350bp product (include 25 nucleotides for primer tails) with the target site in the middle of the amplicon. An ideal amplicon size is between 200-300bp. Follow standard criteria for primer design: 18-21 nucleotides long with 40 to 60% GC content, at least 3 of the last 6 nucleotides as C or G and the 3' nucleotide to be a C or G.

To the 5' end of amplicon-specific primers, add tail sequences as below:
M13F: TGTAAAACGACGGCCAGT
Pigtail: GTGTCTT

Example:

In this amplicon marked by yellow highlights for forward and reverse primers, green highlight marks the target site and blue highlights mark the translation start site and splice site.

AGAGTTAGTGGACGCAAAGTGTGTAGTGGAGAGAAGTGTGTTTAGTTAGTAAAGAAGCT
GTAGCCATGGCCCCTAGCACACAAGAGGACGATACCGTCTCCGGTATACGGAAAGGCATA
CGGGCCATTCTGCTCGGGCCGCCCGGCGCGGGGAAGGGAACGCAGGTGAGAGTTTATCCA
AAGGGCCGCGTGGCTCATTCTGAAGCGGGATAGTGCTCTGGGTTATTGAGGTGAA

Primer sequences to be ordered are:
Forward primer:
TGTAAAACGACGGCCAGTAGAGTTAGTGGACGCAAAG
Reverse primer:
GTGTCTT TTCACCTCAATAACCCAGAG
Product size: 234bp+ 25bp = 259bp

In addition, order an M13F primer with fluorescent tag. We normally use FAM or HEX tags.
M13F-FAM primer: 5'-FAM-TGTAAAACGACGGCCAGT-3'
M13F-HEX primer: 5'-HEX-TGTAAAACGACGGCCAGT-3'

It is important to test primers using a panel of WT DNA from 4 to 8 fish of the same strain. A single robust peak (>1000 amplitude) is expected and detection of 2 peaks of similar amplitude in some or all of the samples indicates an in/del SNP. Sequence PCR product from these samples to identify the in/del and design new set of primers to avoid it.

2. Collection of Tissue (Fin clips or Embryos) for DNA extraction

For founder screening, cross potential founders with wildtype fish and collect embryos at 48-72hpf . For ZFNs and TALENs, we screen 60 embryos/founder by pooling 4 embryos/well. This allows screening of 6 founders/plate. For CRISPRs, due to their higher efficiency, we screen 7 individual embryos/founder, thus screening 12 founders/plate. The remaining wells are used for wildtype and no template PCR controls. Similarly, adult fish from founder out-crosses or heterozygous in-crosses are genotyped by fin clips.

- Use 96-well PCR plates to collect tissue for DNA extraction, making sure to place it at the bottom of the wells for efficient DNA extraction. Care must be taken when transferring embryos to avoid excess egg/system water in the wells (upto 10ul of water will not affect DNA extraction).
- If embryos are in chorion place them in -20^{o}C freezer for 15 minutes to break chorions to allow extraction solutions access to the embryo.

3. DNA Extraction

Our method is optimized to work with DNA extracted using the Extract-N-Amp kit (Sigma) as described below. However, DNA extracted by other methods, such as Qiagen's DNA from Blood and Tissue prep kit also work equally well.

All steps are carried out at room temperature (RT) unless noted.
Volumes below are for fin clips or pools of upto 4 embryos/well. For individual embryos of 48-72hpf, use ½ of the volumes of reagents for each step.

1. Add 50ul of Extraction Solution to sample.
2. Add 12.5ul of Tissue Preparation Solution. Mix well.
   Note: 62.5 ul of pre-mixed Extraction Solution and Tissue Preparation Solution in a 4:1 ratio can be added to combine steps 1 and 2.
3. Incubate at RT for 10 minutes.
4. Incubate samples at 95°C for 5 minutes.
   Note: Tissue will not be completely dissolved at the end of the incubations. This is normal and will not affect performance.
5. Add 50ul of Neutralization Solution B to sample and mix by vortexing.
6. Store DNA at 4°C for up to 2 weeks, -20^{o}C for longer periods or use immediately for PCR.
7. Make 1:10 dilution (5ul DNA: 45ul H2O) of DNA to use for fluorescent PCR reaction. **Critical step** for this method of DNA extraction to remove PCR inhibitors from DNA for efficient amplification.

4. Setting up fluorescent PCR Reaction

1. Make "PCR Brew" (to make 5mL: This can be scaled up)

794ul	10x PCR Gold Buffer (contains no MgCl2)
794ul	25mM MgCl2
198.75ul	10mM dNTP mix
3.15ml	H2O

63.5ul Taq Gold

Aliquot 990ul of brew into tubes and store at -20°C

2. Make Primer mix (all primer stocks are prepared as 100uM)

Forward primer (M13F tailed)	5ul
Reverse primer (pig tailed)	5ul
M13F-FAM or M13F-HEX primer	5ul
TE pH8.0	485ul

3. Set up PCR reaction as follows (Note: This mix is sufficient to set-up two 96-well plates, scale up or down depending upon the number of samples)

a. Thaw 1 tube of PCR brew (990ul) and add 55ul of primer mix.
b. Put 5ul of "brew + primer mix" into each well.
c. Add 1.5ul of diluted DNA and place in thermocycler.

PCR conditions:
94°C for 12 min
35 cycles of
94°C for 30s
57°C for 30s
72°C for 30s
72°C for 10 min
4°C forever

5. Fragment analysis of PCR products by capillary electrophoresis

1. Prepare size standard dilution as 1:50 dilution of ROX400 size standard in HiDi formamide (Note: for PCR products of 375 - 450bp range, use ROX500 size standard)
2. Mix diluted size standard and PCR product as below:
   2 ul PCR product
   10ul ROX400/ HiDi mixture
3. Denature for 5 minutes at 95°C. **(CRITICAL STEP)**
4. Run on ABI capillary electrophoresis Genetic Analyzers, such as 3100, 3130 or 3130xl.
5. Process .fsa files using GeneMapper or Genescan and Genotyper software packages (Applied Biosystems). Always include a wildtype sample as control. If mutant peaks are detected, size of the insertion or deletion can be estimated from the size of the mutant peak. Heterozygous samples can be sequenced to determine the exact sequence of the mutation. Often, the mutations are complex in nature, e.g. deletion of 7bp observed by PCR may actually be deletion of 10bp with insertion of 3bp.

Example of fragment analysis data:
In this example, pools of embryos screened from 2 founders: 18F and 19F. Founder 18F transmits at least 4 different mutations (highlighted in different colors), whereas founder 19F does not transmit any mutations. Therefore, founder 18F is selected as germline transmitting founder with 4bp, 13bp and 19bp deletions and 1bp insertion. Of these, 19bp insertion seems to be most frequent as it is seen in 3 pools.

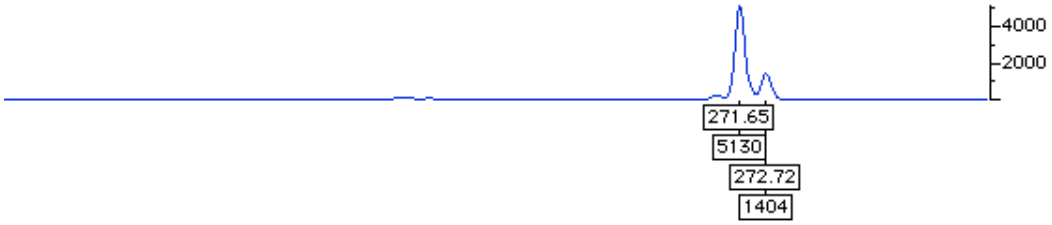
Note: When screening pooled embryos, peak height of wildtype peak is more than the mutant peaks and depends on the number of mutant embryos in the pool.
For single embryos or fin clips: 2 peaks of almost similar peak height are observed.

Please Reference this paper when using this protocol:

Sood, R., Carrington, B., Bishop, K., Jones, M., Rissone, A., Candotti, F., Chandrasekharappa, S.C. and Liu, P. (2013) Efficient methods for targeted mutagenesis in zebrafish using zinc-finger nucleases: data from targeting of nine genes using CompoZr or CoDA ZFNs. *PLoS One*, 8, e57239.

Sample Comment	Peak 1	Peak height 1	Peak 2	Peak height 2	Peak 3	Peak height 3
GL412-18F-p1	271.57	7187				
GL412-18F-p2	271.53	5082				
GL412-18F-p3	253.18	901	271.54	4944		
GL412-18F-p4	271.58	5036				
GL412-18F-p5	271.56	6462				
GL412-18F-p6	259.18	865	271.61	4627		
GL412-18F-p7	271.54	4544				
GL412-18F-p8	259.18	790	267.46	447	271.56	4238
GL412-18F-p9	271.62	6959				
GL412-18F-p10	271.55	5605				
GL412-18F-p11	253.18	694	271.57	3554		
GL412-18F-p12	271.61	5994				
GL412-18F-p13	271.58	5908				
GL412-18F-p14	271.56	5381				
GL412-18F-p15	253.24	573	271.64	3683		
GL412-18F-p16	271.65	5130	272.72	1404		
GL412-19F-p1	271.54	6212				
GL412-19F-p2	271.54	5545				
GL412-19F-p3	271.58	6603				
GL412-19F-p4	271.5	5817				
GL412-19F-p5	271.52	6638				
GL412-19F-p6	271.52	4909				
GL412-19F-p7	271.55	4632				
GL412-19F-p8	271.56	5376				
GL412-19F-p9	271.54	7682				
GL412-19F-p10	271.53	6455				
GL412-19F-p11	271.55	5905				
GL412-19F-p12	271.58	6615				

GL412-18F-p16

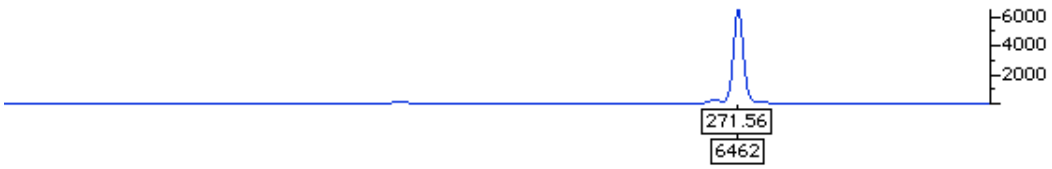


GL412-18F-p2

GL412-18F-p3

GL412-18F-p4

GL412-18F-p5



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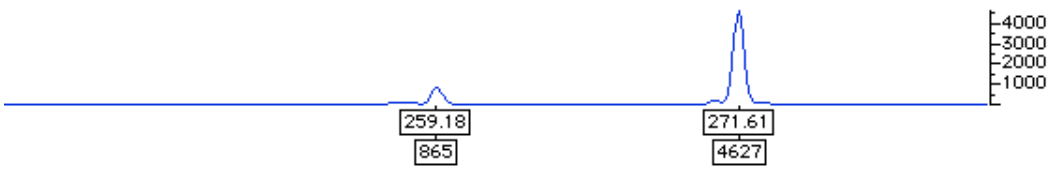

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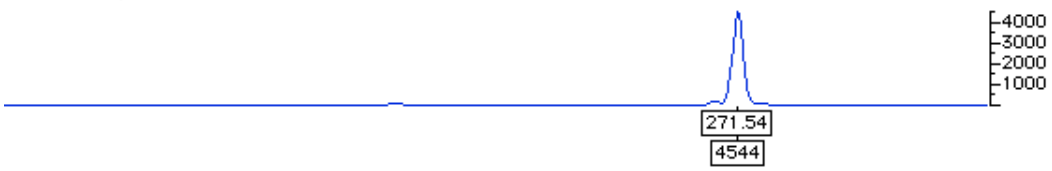

GL412-18F-p8